Animal Models of Chemotherapy-Induced Mucositis:

Translational Relevance and Challenges

Per T. Sangild\textsuperscript{1,2,3}, René Liang Shen\textsuperscript{1}, Peter Pontoppidan\textsuperscript{1,2}, Mathias Rathe\textsuperscript{3}

\textsuperscript{1}Comparative Pediatrics and Nutrition, University of Copenhagen, DK-1860 Frederiksberg C, Denmark; \textsuperscript{2}Department of Pediatrics and Adolescent Medicine, Rigshospitalet, DK-2100 Copenhagen Ø, Denmark; \textsuperscript{3}Hans Christian Andersen Children's Hospital, Odense University Hospital, DK-5000 Odense C, Denmark

Running head: Animal Models of Chemotherapy-Induced Mucositis

*Corresponding author: Per Sangild, Comparative Pediatrics and Nutrition, Faculty of Health and Medical Science, University of Copenhagen, DK-1860 Frederiksberg C, Denmark; Tel +45 26 16 05 30; E mail pts@sund.ku.dk.
Abstract

Chemotherapy for cancer patients induces damaging tissue reactions along the epithelium of the gastrointestinal tract (GIT). This chemotherapy-induced mucositis (CIM) is a serious side effect of cytotoxic drugs and several animal models of CIM have been developed, mainly in rodents and piglets, to help understand the progression of CIM, and how to prevent it. Animal models allow highly controlled experimental conditions, detailed organ (e.g. GIT) insights, standardized, clinically-relevant treatment regimens and discovery of new biomarkers. Still, surprisingly few results from animal models have been translated into clinical CIM management and treatments. The results obtained from specific animal models can be difficult to translate to the diverse range of CIM manifestations in patients that vary according to the antineoplastic drugs, dose, underlying (cancer) disease and patient characteristics (e.g. age, genetics, body constitution). Another factors that hinder the direct use of results from animals are inadequate collaboration between basic science and clinical science in relation to CIM. Here, we briefly describe CIM pathophysiology, particularly the basic knowledge that has been obtained from CIM animal models. These model studies have indicated potential new preventive and ameliorating interventions, including supplementation with natural bioactive diets (e.g. milk fractions, colostrum, plant extracts), nutrients (e.g. PUFA, SCFA, glutamine) and growth factor peptides (e.g. TGF, GLP-2), as well as manipulations of the gut microbiota (e.g. pre-, pro- and antibiotics). Rodent CIM models allow well-controlled, in-depth studies of animals with or without tumors while pig models easier make clinically-relevant treatment regimens possible. In synergy, animal models of CIM provide the basic physiological understanding and the new ideas for treatment that are required to make competent decisions in clinical practice.

Key words: Mucositis, chemotherapy, pig, rat, mice, inflammation, toxicity, intestine
Clinical consequences of CIM and the potential of animal model research

Chemotherapy and radiation therapy may induce mucositis, a common adverse effect of antineoplastic treatment that has severe consequences for the morbidity and mortality of cancer patients (36, 49, 172). The toxicity of antineoplastic drugs is not specific to malignant cells and thus all actively proliferating tissues may be affected by chemotherapy. The rapid proliferation of intestinal epithelial cells, together with the complex immunological role and interaction with the gut microbiota, makes the gastrointestinal tract (GIT) particularly vulnerable (171). Consequently, chemotherapy for cancer patients may lead to chemotherapy-induced mucositis (CIM). Certain types of radiation therapy induce symptoms similar to CIM, however in this review we focus on the GIT responses to chemotherapy and potential interventions to prevent and reduce these complications.

Broadly defined, CIM is damage to mucous membranes caused by antineoplastic therapy, leading to structural, functional and immunological changes. Previously, CIM was considered mainly an epithelium-related condition resulting from direct damage to dividing epithelial cells and loss of renewal capacity. It is now recognized as a much more complex condition that involves all cells and tissues of the mucosa (121, 171), interacting with the luminal environment and a systemic inflammatory responses (200). CIM may be subdivided into oral mucositis (OM) and intestinal mucositis (IM), two closely interacting clinical diagnoses. The majority of previous research has focused on OM, partly due to the difficulties in direct study of GIT tissues from cancer patients beyond their oral cavity (4, 110, 171). Because IM cannot be visually inspected (in contrast to OM), IM is described primarily via clinical symptoms or biomarkers (84, 111).

CIM is one of the most debilitating adverse effects of chemotherapy, being a principal reason for pain and reduced quality of life during cancer treatment (6, 36, 96). In addition to the GIT structural effects, CIM may compromise nutrient and fluid absorption, GIT endocrine functions and the intestinal
barrier. This may cause malnutrition and bacterial translocation and contribute to the systemic inflammation with consequences for the morbidity and mortality of cancer patients (19, 110, 162, 200).

The exact CIM symptoms vary according to a range of interacting factors, such as age, gender, genetics, body constitution, background disease, pharmacodynamics and the type and dose of antineoplastic drug.

Due to the complex and diverse clinical symptoms of CIM, and the importance of reducing this toxic complication of chemotherapy, it is highly relevant to develop animal models to help understand treatment toxicities and explore new ways to manage adverse effects of chemotherapy. Like in other areas of medicine, the goal of CIM translational research is to combine scientific disciplines, resources and techniques to improve preventive, diagnostic or therapeutic strategies (39). There are limited options to perform well-controlled clinical studies in chemotherapy patients, due to many ethical restrictions and risks associated with invasive procedures. In-depth studies at the structural, cellular and mechanistic level require multiple tissue samples and biopsies. Some of these concerns may be even higher for children in chemotherapy. Consequently, scientific knowledge about CIM in humans is obtained mainly from studies of the oral cavity in adults while studies of IM are fewer (27, 98, 138, 213). Animal models of CIM allow tissue sampling along the entire GIT in response to different genotypes, environmental factors and drug regimens in tumor-bearing animals or animals that are healthy before chemotherapy. Until now, most preclinical studies of CIM have been carried out in adult rodents (26, 203). Model studies in larger animals, such as pigs, may better allow for clinically-relevant supportive treatments (e.g. nutritional, microbiota) and studies during early GIT development. On the other hand, large animal models lack the potential to study tumor-bearing or genetically-manipulated animals.
This review serves to highlight some of the areas where animal models have contributed most
to new knowledge about CIM, its biomarkers and ideas for treatment. Following an introduction to the
most commonly used models in rodents and pigs, we describe how animal models have contributed to
the current understanding of CIM pathophysiology, to validate CIM biomarkers and to explore new
CIM treatments, including for children. Only a range of animal models is likely to provide the
knowledge that will advance both basic science and clinical management of CIM.

The challenge of translational research in relation to CIM

Relatively few scientific discoveries in animals have been translated into clinical diagnostic
tools or treatments for CIM in human patients. A number of biological, technical, practical, educational
and societal aspects may explain this (26, 165). First, the occurrence of CIM in relation to treatment of
cancer diseases may result from close interaction between the neoplasm itself and the specific
chemotherapy treatment regimen, probably making tumor-bearing animal models most relevant in CIM
research. However, it is a challenge to mimic both the pathophysiology of a specific type of tumor in
humans, together with the consequences of specific clinically-relevant treatment regimens. Further,
tumor growth and physiology, as well as the treatment responses, may differ widely depending on
patient variables such as age, gender, genetics and body constitution. This, coupled with the wide
diversity of different chemotherapy regimens, and associated supportive treatments (antibiotics,
nutritional or other), show how difficult it is in a single animal model to accurately mimic a specific
group of human cancer patients. In CIM research, it is highly relevant that chemotherapy regimens are
also studied in healthy animals, independently of the interacting effects of a tumor, because this allows
for more direct interpretation of the specific chemotherapy effects. Considering such aims, cell, tissue
and organ models of CIM are relevant to understand basic mechanisms of CIM and its possible
treatments (53, 74, 94, 182), as they allow study of chemotherapy effects in isolated systems, independent of interacting factors, such as other cell types, systemic influences and the gut microbiota. On the other hand, the multifactorial nature of CIM makes it very difficult to conclude from such isolated in vitro studies to the relevance for intact human patients, making animal models, despite their limitations, a natural component of CIM research with clinical implications.

It is beyond the scope of this review to discuss the limitations of animal models in detail but Figure 1 shows some key challenges that make it difficult to translate basic science, using animal models, to clinical management of CIM in human patients. Still, scientific evidence from animal studies improves the general biological understanding of CIM and thereby provides a foundation for new treatments, if not short term, then more long term. Each animal study provides an opportunity to learn more about the factors leading to CIM, and healing from CIM, and this may contribute to understand the biology of disease conditions far beyond CIM. Translational research, using animal models, functions as a "bridge" that connects basic science with clinical diagnosis and treatment (Figure 1).

The difficulties in translation may relate partly to species differences in genetics and immunology. In one study, acute inflammatory stress from different etiologies resulted in similar genomic responses in humans, while the corresponding responses in murine models correlated poorly with human conditions (165). Immunological responses locally in the GIT and systemically, and the interaction with the luminal GIT microbiota, are central in CIM and these key factors differ among different mammals. Thus, immunity-microbiota interactions, genotype differences, the inbred nature of most rodent models, the high variability of human cancer diseases and heterogeneity of cancer patients are some of the factors that may explain the low success rate of clinical trials for drug candidates, identified in rodent model studies (165). This underlines the need for animal models with a high degree
of translatability and this is particularly important when testing interventions targeted towards the complex physiological and microbiological environment of the GIT. An overview of the most commonly used animal models in CIM research is shown in Table 1, together with some of the typical cytotoxic treatments and endpoints measured.

The high variability in the anatomy and physiology of the GIT among mammalian species is closely related to the different dietary habits of each species (e.g. omnivores, carnivores, herbivores). Clearly, this influences the response of the various GIT tissues to cytotoxic drugs and related treatments (e.g. enteral nutrition, anti-, pre- and probiotics). On this background, it is relevant to supplement some of the advantages known from rodent models (e.g. easy handling, low price, access to genetic manipulations, widely available analytical technology) with animal models that are more similar to humans in their anatomy, physiology, immunology and microbiology of the GIT and more easily allow manipulation of factors that affect the local GIT environment (e.g. parenteral/enteral nutrition, gut microbiota interventions). Animal models in rabbits, cats, and dogs have been used but with limited focus on IM (Table 1). Still, all types of animal models are required, as they contribute with unique opportunities to investigate different aspects of the complex conditions in the GIT in relation to CIM. Any model will have strengths and weaknesses and implementation of standardized treatments in clinical settings should always be based on well-designed, randomized controlled clinical trials, emphasizing the need for interdisciplinary collaboration when developing and testing potential treatments, from cells, over animals to human patients (Figure 2).

Before discussing the biology of CIM in specific animal models, it is important to mention the ethical dilemmas involved in CIM research. Inducing CIM in an animal causes variable degrees of pain, similar to the condition in human patients subjected to chemotherapy. In experimental CIM research, treated animals typically need intensive clinical care to avoid unnecessary suffering and high
mortality. Euthanasia, following carefully defined humane endpoints, may be required for individuals in CIM animal studies. Thus, CIM animal model research is challenging from an animal ethics perspective and careful judgment of the expected relevance and benefits of the results, relative to animal welfare considerations and risks, must be made by relevant authorities, as well as by the involved researchers.

**The pathophysiology of CIM, as supported by results from animal models**

To a large degree, the current understanding of CIM is based on in vitro cell studies and rodent models of CIM. The 5-phase model, originally presented by Sonis et al. (169), was developed to explain OM but it has been extended to include IM and CIM in general (171). According to this, the five stages of CIM are initiation, messenger signaling, signal amplification, ulceration, and healing. These five phases describes the pathophysiological sequence of GIT toxicity, starting with clonogenic cell death and the release of reactive oxygen species, progressing through the activation and amplifications of biological pathways leading to ulcer development, and eventually healing. The 5-phase CIM model suggest a primary response and a secondary inflammatory response, and fits well with clinical findings in HSCT patients, where two peaks in inflammation is often seen, first during the chemotherapy conditioning and then during clinical CIM (149). The pathobiology of CIM has been related to many GIT factors, such as mucins and disruption of the microbiota leading to dysbiosis (144, 156, 180, 181), the pattern recognition receptors involved in tissue homeostasis and immune tolerance (e.g. toll like receptors, specifically TLR-2, -4 and -9) (63, 95, 184, 207), and tight junction proteins with consequences for intestinal barrier function (18, 76). In relation to CIM, increased release of pro-inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor (TNF) and interleukin 6 (IL-6) (76, 122, 123) are mediated partly by early up-regulation of NF-κB and possibly maintained by
further NF-κB activation and activation of injury pathways (4). The matrix metallopeptidases (MMP) and their regulators (2, 4, 28) are also important, and kruppel-like factors (KLF), specifically KLF 4, are known to be involved in maintaining intestinal epithelial homeostasis (180, 181). The chemokines belonging to the CXC motif ligand family and their receptors (78, 126), as well as bone morphogenic proteins (BMP) involved in epithelial-mesenchymal signaling and intestinal stem cell activity (42), has also been implicated in CIM pathophysiology. The list of factors is continuously growing and questions remain regarding the highly dynamic processes affecting different regions of the GIT at different time points. More research is still needed to elucidate the temporal sequence of events and the mutual interplay of different factors in response to different drugs and treatment regimens in both animals and humans of different ages (79, 93, 123).

The availability of tumor-bearing and genetically-manipulated rodents has lead to great progress in the basic understanding of CIM pathophysiology but the GIT and tumor responses to different drugs (e.g. MTX, 5FU, irinotecan, doxorubicin) vary widely (203). Rodent studies have been critical for our understanding of the role and control of enterocyte turnover in CIM, and recovery from CIM, and Wnt/β-catenin signaling has been documented as a critical factor (182). Likewise, rodent studies have shown that a key element in CIM is mitochondrial coordination of apoptosis signaling pathways, partly via bax and bcl-2 proteins (27, 185). It remains however, that the key elements in CIM progression and recovery may be highly species-, region-, time- and drug-dependent, making explorative omics analyses attractive. Microarray analyses of tissue obtained from the oral cavity of humans have confirmed the chemotherapy effects on some of the single genes identified in the GIT of animal models, including factors such as argininosuccinate synthase, notch and zinc transporters (138).

Correspondingly, analyses of the intestinal transcriptome in piglets following two different CIM-inducing chemotherapy regimens revealed a subset of differentially regulated genes related to defense...
response and innate immunity (155). Comprehensive understanding of CIM pathophysiology is difficult without animal models that allow serial testing of clinically-relevant treatment regimens, followed by access to the entire GIT during the various stages of CIM.

Models of CIM in rodents, strengths and limitations

In hamsters, an oral cheek pouch has provided a unique opportunity to study OM (174) and mucositis has been induced by injection of 5-flourouracil (5-FU) for three days and by scratching the epithelium on days 1-3. Studies in this animal model generated the important 5-phase pathophysiological model of CIM, constituting the foundation for the current understanding of CIM (26, 169, 170). In addition to hamsters, a series of rodent models with various treatment regimens (drugs, doses, length of treatment and follow-up period) have been developed (26, 203). Murine models of CIM have evolved for chemotherapeutic agents such as irinotecan (91), methotrexate (MTX) (44), cyclophosphamide (212, 222), 5-FU (12, 88), cisplatin (91), melphalan (31), doxorubicin (43, 95), cytosine arabinoside (Ara-C) and vincristine (VCR) (10), as well as combinations of irinotecan and 5-FU (146) among others.

As mentioned earlier, results from studies on healthy animals cannot adequately answer biological questions of CIM that depend on interactions with a solid or hematologic neoplasm. In rats, CIM severity is affected by tumor burden (66) and studies show that proinflammatory cytokines, released by tumor tissue (e.g. IL-1β, IL-6, TNF-α, INF-γ), may affect food intake and energy expenditure leading to cancer cachexia (16). However, implanted tumors in rodents often comprise a greater fraction of total body weight, compared with humans, resulting in pronounced effects on whole body and GIT metabolism (15). This aspect complicates translation of CIM results in tumor-bearing rodent models and may explain some discrepancies between animal and human studies in studies of
nutrition, chemotherapy and tumor growth (16, 192). Numerous case-studies are available on CIM in pet animals having spontaneous cancer development and subsequently receiving chemotherapy, especially cats and dogs (41, 209). While these spontaneous CIM animal models are important, their limited scale and diversity of different tumors, combined with the lack of appropriate controls, limit the translational value of results for humans.

In rats, development of the mammary gland adenocarcinoma model is one of several good models that allow simultaneous assessment of tumor growth and the severity of CIM associated with chemotherapy (97, 203). It has been applied to investigate the mucotoxic effects of important and widely used antineoplastic drugs such as MTX (27, 67), irinotecan (3, 28, 66, 68, 179) and 5-FU (123, 180). This model has contributed greatly to the understanding of various aspects of CIM (203). While rodent models have been used to test many different agents with promising antimucotoxic properties, only few results have been translated into novel clinical treatments for humans. Some successful examples are palifermin (KGF) (54, 69), probiotics (29), laser therapy (115) and amifostine (35) that are indicated as part of current treatment recommendations for patients receiving chemotherapy in adults (113). Numerous other interventions against CIM have been evaluated in rodents, including the macrolide antibiotic, clarithromycin (212), grape seed extract (34), IL-11 (71, 173), celecoxib (92), dexrazoxane (143), short chain fatty acids (56, 152), IL1Ra (205), cathepsin inhibitors (5), emu oil (119), GLP-1 and -2 (102, 103), Chinese herbal medicine (73, 120), iseganan (125), TGF-β2 (17), TGF-α (187), St. John’s wort (86, 87), glutamine (184), arginine (118), leptin (183), fermented mulberry leaf extract (140), milk bioactives with/without other compounds (23, 24, 37, 85, 195), oral insulin (109), dexamethasone (115), and feeding strategies such as minimal enteral feeding (108). Some interventions, such as that of clarithromycin (221), celecoxib (92, 114), Chinese herbal medicine (89), whey protein concentrate (147), dexamethasone mouth wash (157) and TGF-β2 (43), have been
subject to trials in humans, but have not yet resulted in general recommendations. Some authors suggested beneficial effects of IL-11 therapy (48) but others reported a high rate of toxicity and mortality in a small group of patients given the same dose of rhIL-11 (11). Glutamine supplementation has been evaluated in several clinical studies but the results are inconsistent and current guidelines for CIM treatment do not include glutamine recommendations (113, 215). The above mentioned long list of CIM interventions tested in rodent studies illustrates the problem in translating from bench to bedside in CIM research (26).

Models of CIM in pigs, strengths and limitations

The pig is often argued to be a superior biomedical research model due to its higher genetic, anatomical and physiological similarity with humans (136, 163), relative to rodents. The pig GIT metagenome has also recently been documented to be relatively similar to the human GIT metagenome (214) which is valuable when studying treatments and drugs that affect the inflammatory status of the GIT mucosa and its interaction with the microbiota (81). Furthermore, piglets may have certain advantages for studies of GIT conditions and diseases for pediatric patients, including pediatric CIM and necrotizing enterocolitis (141, 158, 159). The similarity in size and physiology to humans allow pigs to be used for experimental procedures that may not be feasible in rodents, such as various forms of surgery (52), multiple catheterizations and complex, clinically relevant supportive therapies (e.g. antibiotics, antiemetics, analgesics, hyperhydration, uroprotective drugs) (150).

Post-puberty growing pigs are similar in body mass and size to humans allowing experimental, clinical and surgical procedures in pigs that are similar to those for adult human patients. However, the high genetic potential for excessive muscle growth in domesticated pigs may affect the responses to cancer and therapeutic drugs. Models of bone marrow transplantation and CIM have been developed in
mini pigs (47, 168) and these pigs show a body growth pattern that is more similar to that in humans. Due to their smaller size, mini pigs reduce the costs related to housing and supportive care procedures and drugs, but still, they remain much more expensive to use in CIM research than rodents. Using normal production pigs, doxorubicin (adriamycin) has been investigated in weanling (4-8 week-old) pigs, causing multi-organ toxicities with clear signs of both IM and OM, reduced appetite and diarrhea (130, 201). High doxorubicin doses are associated with severe diarrhea, rapid clinical deterioration, multiorgan complications and sepsis-related mortality. In these studies, the GIT lesions were primarily located to the colon while the small intestine was only mildly affected by atrophy and inflammation. Survival was generally better in younger versus older pigs and systemic antibiotics had limited effects. In another study in young pigs, 5-FU treatment resulted in wasting, appetite reduction, diarrhea, vomiting and intestinal mucosal atrophy and dysfunction (128). The antioxidant defence system was impaired and blood leukopenia and immunosuppression in the intestinal lymphocyte population were found, without notable mortality. Low experimental mortality would be an important aim from an ethical and experimental perspective.

Recently, models in young pigs (Figure 3) have used non-myeloablative doxorubicin to reflect a common initial treatment in pediatric acute lymphoblastic leukemia (ALL), the most frequent cancer diagnosis during childhood (90) to induce CIM in 1-2 week experiments. In weanling pigs, histological examinations confirmed damage to the intestinal mucosa, together with reduced lactase activity and glucose uptake after a single dose of the drug, and it increased the chloride secretion and circulating levels of TNF (130). At 5-6 days after doxorubicin treatment, the intestine appears to be in a regenerative state with minimal effects seen on villus structure although enterocyte functions may remain compromised and be responsible for continuous wasting and diarrhea, increased permeability and increasing plasma levels of C-reactive protein (CRP). When even younger (milk-fed) pigs are used
(166, 167), doxorubicin treatment reduces the white blood cell (WBC) counts, hexose absorptive function, plasma citrulline, weight of the intestine, colon and spleen, and it increases the gut permeability and plasma CRP levels. In these studies, limited or no effects were observed for digestive enzymes, proinflammatory cytokines, or tight-junction proteins in the intestine. Possibly, the younger age of the animals, and thereby the more immature stage of a milk-fed intestine, with slower enterocyte proliferation and limited bacterial colonization, make the GIT better able to tolerate chemotherapy. Alternatively, an immature intestinal structure, function and immunology, including bacterial defense mechanisms, may predispose the intestine to damage and translocation. When a more intense myeloablative chemotherapy, based on busulfan and cyclophosphamide, were used in an 11-d protocol for young milk-fed pigs, the treatment markedly reduced WBC platelets and induced both OM and IM, as evidenced by diarrhea together with reduced intestinal weight, villous heights and brush border enzyme activities (150). In these studies, a proportion of the animals had to be euthanized before 11 days, reflecting the high level of toxicity in this model. It resembles the highly intensive cytotoxic regimens administered prior to hematopoietic stem cell transplantation (HSCT) in children, with hallmark clinical signs of CIM toxic complications being OM, wasting, appetite loss, diarrhea, vomiting and increased intestinal permeability. In such animal model studies, it is critical to understand the temporal progression of GIT toxicity and the time period when a given treatment would be most effective to reduce or protect against CIM.

CIM diagnosis and biomarkers supported by animal models

To validate animal models of CIM, it is important to use scoring systems that mimic the human condition. However, the full and direct availability of organs from experimental animals, including the entire GIT, allows for many other structural and functional parameters to be evaluated when assessing
the severity of toxicity in animal studies. The many different clinical CIM scoring systems reflect the subjective nature of CIM diagnosis in human patients (e.g. patient-reported ability to eat/drink/swallow, speaking problems, abdominal discomfort, nausea, vomiting, diarrhea). More objective parameters have been incorporated in some grading systems (e.g. presence of fever, infections, need for parenteral nutrition) but clearly, these remains affected by a number of variables other than the chemotherapy itself. In well-controlled animal studies, the number and effects of such interacting variables are fewer and thus, less likely to affect the CIM evaluation. Further, animal models may help to validate clinical scoring systems by correlating various biomarkers with the presence of both structural and functional GIT effects. Most clinical CIM scoring systems are not validated for pediatric patients and it is possible that children show quite different symptoms of CIM than adults due to their developing GIT structure, microbiota and immune responses to chemotherapy.

In adult oncology, at least 20-50% of patients receiving standard dose chemotherapy develop OM, with a frequency and severity of symptoms highly dependent on the type and dose of chemotherapy and the score system used to evaluate mucositis (164, 170, 171). The responses to different antineoplastic drugs may be age-dependent and both lower and higher incidences have been reported in pediatric oncology (57, 133, 154). Higher incidences in children may be explained by the frequent use of high-dose, multidrug regimens for this population (6, 164). Among drugs commonly associated with OM in children are alkylating agents, such as busulfan and cyclophosphamide, antimetabolites such as methotrexate, 5-FU and cepacitabine, and anthracyclines, such as doxorubicin (6, 154, 164). As in adults, the risk of IM has received less attention compared with OM, but studies indicate that IM is present in 30-60% of children receiving chemotherapy (110, 154). Considering the challenges in accurately assessing CIM in humans, especially IM, the use of biomarkers is critical for more accurate diagnosis or to supplement clinical assessments (111).
Chemotherapy generally causes shortening of the villi, with continued migration and cell sloughing while mitosis is reduced, leading to marked changes in the steady state of the epithelium (93). Time-dependent villous atrophy is associated with CIM in both rodents and piglets and examining intestinal morphology is highly relevant in preclinical models (95, 150). Chemotherapy may also induce other histopathological signs of damage and several scoring systems incorporate these (44, 211).

Further, the relative proportions of various brush border enzyme activities throughout the small intestine can be used to reflect mucosal digestive function (150, 167). Animal studies have documented chemotherapy-induced increase in tissue levels of pro-inflammatory cytokines (76, 123, 148), damage to tight junction proteins and intestinal integrity (132), and impaired nutrient absorption (60). In few human studies, the lactose and sucrose breath tests have been used to assess mucosal digestive function but the tests are unlikely to be sensitive and specific enough for wide application as biomarkers of CIM in clinical practice (111). Animal studies using rodents and pigs would help to further validate these biomarker tests based on disaccharide digestion (60, 167). Chemotherapy may directly or indirectly also affect GIT motility and the associated enteric nervous system, both short and long term (50, 131).

Measures of gastric emptying rate and intestinal transit time can be obtained from both patients and animal models, but only animal models allow detailed analyses of the structural and functional damage to the enteric nervous system that may lead to dysmotility, constipation and/or diarrhea (176). This is an overlooked research area and animal models with a great similarity to humans in their GIT enteric nervous system and motility patterns are required to study this in more detail.

The relation between chemotherapy, neutropenia, CIM and infections in cancer patients is well recognized and antibiotics treatment is critical for survival (160). CIM severity correlates closely with infection sensitivity, indicating that CIM directly increases systemic infections (49, 83, 172). The systemic immune dysfunction induced by chemotherapy increases the risk of infections, with
bacteremia occurring when WBC counts are lowest, and coinciding with a peak in CIM (19, 65, 82, 83, 197). CIM may disrupt the barrier functions of the intestine and some infectious complications appear to be related to the resident oral and gut microbiota translocating to the bloodstream (40, 64, 82, 134). Thus, commensal bacteria detected in the bloodstream, such as the coagulase-negative staphylococci, may originate not only from skin surfaces but also from mucosal sources in the GIT (40). The role of the gut microbiota in these inflammatory CIM responses may be most important after the initial apoptotic phase, as demonstrated in doxorubicin-treated, germ-frem mice (156), and show the relevance of interventions that manipulate the gut microbiota. During chemotherapy many patients also experience episodes of febrile neutropenia that cannot be explained by an infection (14, 177). The cause of fever in such patients is not clear, but it is likely due to systemic inflammation induced by the chemotherapy itself during its initial phase. Temporal associations among CIM, systemic inflammation and fever have been demonstrated, independent of diagnosed infections, leading to the term, febrile mucositis (19, 149, 197, 199).

Several innate immune factors have been proposed as possible determinants of infectious morbidity in patients receiving chemotherapy. Among these are mannose-binding lectin (MBL2), ficolin, TLR-4 (135, 145) and NOD2 (220). Much research has been done for MBL but the evidence for effects of MBL deficiency remains conflicting (7, 46, 55, 62, 101, 105, 116, 139, 142). Similar conflicting results has been obtained for ficolin deficiency as a risk factor (142, 161) (101). It is perhaps unlikely that any single biomarker can effectively identify patients at increased risk and animal models may help validate and guide the selection of relevant soluble factors and gene polymorphisms but also study effects of interacting factors such as age, underlying diagnosis and treatment regimen.

Citrulline, a non-essential amino acid synthesized almost exclusively by the enterocytes, is currently considered a relatively reliable measure of IM damage (111) and plasma citrulline levels may
discriminate different chemotherapy regimens (20, 72, 84, 111, 127, 198, 202). Low citrulline levels has been associated with the occurrence of bacteremia and increased production of pro-inflammatory cytokines following chemotherapy treatment (72, 82, 149). The value of citrulline as an indirect blood marker of IM-associated mucosal atrophy has been validated in both piglets (148, 166, 167) and rodents (58-60). Citrulline levels may also relate to intestinal fatty acid-binding protein (I-FABP) and ileal bile acid-binding protein (I-BABP) levels in plasma, released from dying villous enterocytes. Animal studies may help to validate the initial reports that combined plasma citrulline, I-FABP and I-BABP levels can be used to evaluate CIM following myeloablative chemotherapy (45).

Cytokines and inflammatory markers play key roles in the pathogenesis of mucositis and elevated serum levels of pro-inflammatory cytokines, such as TNF, IL-1β and IL-6 and acute phase reactants, such as CRP, are markers of the inflammatory response induced by various chemotherapeutic agents but cannot be considered specific for mucositis (65, 111). Still, increased CRP levels may correlate closely with severity of intestinal damage in both humans (19) and piglets (166, 167). Other cytokines, such as IL-1, IL-17a and IL-8, may also reflect the inflammatory response related to GIT toxicity (9, 149, 202). Furthermore, the pre-treatment levels of inflammatory mediators may predict the later risk of CIM during treatment (218). Although GIT toxicity and the systemic inflammatory response after chemotherapy are connected, plasma cytokine and inflammatory marker levels are not effective biomarkers of CIM because they may be secreted by multiple tissue sites and cell types in the entire organism in a highly time-dependent manner (111). This lacking specificity of cytokine levels has been confirmed in pig studies where different GIT toxicity levels did not correlate well with circulating or GIT tissue cytokine levels (150, 166, 167).

Increased intestinal permeability induced by chemotherapy is an essential aspect of IM and it is related to the inflammatory and infectious complications associated with toxicity (21, 99, 132).
Different sugar permeability tests have been used to assess intestinal barrier function in clinical studies, although the required fasting period and collection of blood or urine over an extended time period limits the clinical feasibility of the test (65). Regardless, animal studies in both pigs and rats have confirmed that the intestinal permeability to sugars is an effective way to assess this aspect of CIM (150, 167, 188). Direct chemotherapy-related damage to the nutrient absorptive capacity is reported but the evidence is weaker than for intestinal permeability. The sucrose breath test has been used clinically (191) and animal studies have confirmed that sugar absorption tests, especially those combined with sugar digestion (e.g. sucrose and lactose hydrolysis), are effective methods to characterize the functional damage induced to enterocytes by chemotherapy in both rats (58-60) and pigs (150, 167).

Abundance and diversity of the gut microbiota may be reduced in response to chemotherapy in rodents, pigs as well as humans (148, 151, 156, 188). Based on human faecal samples, myoablative chemotherapy decreases the abundance of Firmicutes and Actinobacteria, and increases Proteobacteria, probably resulting in reduced gut capacity for nucleotide, energy and vitamin metabolism, and increased capacity for glycan and xenobiotics metabolism (137, 178). Many patients show decreases in Lactobacilli, Bifidobacteria, Bacteroides and Enterococci while Escherichia coli and Staphylococci may increase (178). In the small intestine, increased Escherichia levels were also observed in CIM piglets, but also increased Lactobacilli, at least short-term (148). It remains however, that the changes to the microbiota may be indirect and secondary to chemotherapy-induced damage of the intestinal epithelum and immune system, disturbed intestinal motility and secretions, together with the associated use of antibiotics. Thus, it should be better clarified to which extent the gut microbiota is a relevant CIM biomarker, alone or in combination with gut microbiota-dependent tests, such as the breath hydrogen test. Interestingly, rodent studies indicate that the microbiota exert highly GIT region-dependent effects on CIM, with the contribution to inflammatory damage highest for the small intestine.
and role in recovery most important for the colon (208). In addition, CIM pathologies are not always microbiota-dependent, as shown in mice (156). Detailed studies in animals could help to clarify specific chemotherapy effects on the microbiota at various GIT sites (e.g. mucosa-associated versus luminal, and intestinal versus colonic/faecal microbiota) and its indirect and direct effects on CIM damage and recovery. On the other hand, it is important to acknowledge that the gut microbiota haboured by different GIT sections differs widely among mammals due to differences in dietary habbits and GIT anatomy and physiology, potentially limiting the translational value of animal studies in this field. Regardless, exogenous manipulation of the gut microbiota (e.g. using anti-, pre-, probiotics) remains to be a promising new tool in limiting clinical CIM (194).

**Interventions for CIM supported by animal models**

Numerous interventions have been proposed and tested for the prevention or amelioration of CIM and animal models have contributed much to the list of possible interventions, including gut- and nutrition-related effects (Table 2). Some updated evidence-based guidelines (70, 113, 217) refer to highly specific human disease settings, and they include suggestions for use of supportive drugs (e.g. amifostine, actreocitide, sucralfate enemas, sulfasalazine), probiotics and oxygen treatment. Specifically for OM, oral cryotherapy, keratinocyte growth factor (KGF) 1, laser therapy, oral benzydamine and systemic zinc are recommended. In the pediatric population, interventions are more limited and no preventive measures for CIM are widely accepted. This highlights the need for continuous research to identify new treatment strategies for CIM based on preclinical models.

The GIT is the focus of CIM toxicity and nutritional interventions and modulations of the gut microbiota are therefore important among the potential management options. The GI mucosa is in direct contact with the microbiota, diet and luminal ligands. The GIT facilitates nutrient transfer but
also specific dietary factors that may promote GI mucosal integrity and protection via suppression of
gut inflammation, antimicrobial effects, and luminal nutrition facilitates mucosal repair (24, 85, 195).
Malnutrition is associated with poor outcomes of CIM (100) but the link and causative factors are not
clear. If the intestine is not severely damaged, enteral nutrition is preferred (16) and studies in rats
show healing effects of even small amounts of enteral nutrition following methotrexate-induced
mucositis (59, 108). For many CIM patients, enteral nutrition via tube feeding becomes necessary and
enteral feeding represent an important therapeutic potential. While parenteral nutrition may improve
the overall caloric and nutrient intake when GIT functions are severely compromised, there is limited
evidence to support the importance of parenteral nutrition for CIM patients, at least short-term (110,
112). In fact, a recent randomized study suggested that fast introduction of parenteral nutrition in
hospitalized, critically-ill children was detrimental (61). Conversely, enteral nutrition may help to
preserve the integrity of the intestinal mucosa and reduce the risk of bacterial translocation through the
stimulation of intestinal growth, enterocyte function and barrier function (77, 110). This is a need for
further studies in animals that allow easy manipulation of enteral and parenteral nutrient intakes before
and after chemotherapy. Possibly, the role of enteral nutrition is more important in early life when the
GIT is still immature and adapted to a milk diet (159). A series of studies in young rodents also suggest
beneficial effects of different milk compounds for CIM. Milk is a natural source of many bioactive
compounds, including growth factors, immunomodulatory compounds and antimicrobials, and milk
factors, given with or without other compounds, protect against CIM (23, 37, 85, 195) (Table 2).
Most of the proposed beneficial compounds in milk are found in much higher concentration in
colostrum than in mature milk (32). In piglets, a diet supplement consisting of cow’s colostrum, a milk
diet particularly rich in immunomodulatory and antibacterial factors, ameliorates the short-term effects
of doxorubicin (130, 166) and also of the more intense myeloablative treatment using busulfan and
cyclophosphamide (148). Thus diet factors may help the general nutritional status but also promote specific GIT mucosal integrity via antimicrobial and endotoxin-neutralizing effects, suppression of gut inflammation and promotion of mucosal tissue repair. Clinical trials of colostrum supplement to children with ALL are ongoing (ClinicalTrials.gov Identifier: NCT01766804), based on colostrum effects reported in other GIT conditions and diseases (153). Other diet supplements investigated for children include honey, olive oil, propolis, beeswax, bee glue, vitamin E, ursodeoxycholic acid, glutamine and TGF-β2 (1, 8, 43, 190, 206) but further studies are required to verify their specific bioactive effects on CIM and as yet, none have resulted in general clinical recommendations (113).

**Translational relevance and challenges of animal models in CIM research**

The greatest overall challenge for animal models of CIM is the difficulty in translating results obtained from current models to the wide range of human patient groups, with varying ages, genotypes, phenotypes, cancer diagnoses, and to treatments covering a wide range of drugs and doses of chemotherapy. Another challenge is a high treatment- and time-dependent morbidity and mortality in experimental animal model studies, even if these complications are clinically-relevant model aspects. This complexity, and a number of additional factors related to animal studies (economics, available facilities, need for genetic manipulations, standard laboratory assays) have made rodents the most commonly used animal model in CIM research (Table 1). Pigs are increasingly used and may add further clinically-relevant treatment regimens, interventions and sampling of biological material, coupled with a presumed closer similarity to humans with regards to GIT anatomy, physiology, immunology and microbiota. Across models, it remains a difficult balance to combine clinically-relevant chemotherapy regimens, and GIT toxicity levels, with acceptable ethical standards and animal care. If biological material cannot be collected from the severely affected animals then this also
precludes conclusions regarding the mechanisms of severe CIM and improved understanding for clinical management.

Preclinical rodent models have focused most on the initiation phase of CIM and often these animal models show regression of symptoms already after 2-4 days. However, patient outcomes may be highly influenced by complications in the GIT at much later time points, coinciding with the nadir in WBC counts and a continuously increasing intestinal permeability, leading to microbial translocation and systemic inflammation. The clinical aspects, as well as the preventive and therapeutic interventions for these later phases, may be most effectively studied in pigs. The exact time frame of the initiation, inflammation and recovery phases of CIM may relate closely to the intestinal cellular turnover and be highly region-, age-, dose-, drug- and species-dependent. This also explains that central mucosal parameters, such as brush-border enzymes and inflammatory cytokines, show quite variable responses in different CIM studies, even using the same animal at a comparable age. It is not possible to describe a universally valid time and dose protocol for doing CIM studies of high sensitivity in animals, and protocols need to be adjusted according to specific phases of the CIM progression, the drug and the dose. In the future, it is critical to use animals to help understand not only short term effects of CIM, but also long-term effects, both locally in the GIT and beyond (e.g. immunological, metabolic, neurological and endocrinological effects). Long-term studies of chemotherapy and CIM require animal models that show a high biological similarity with humans, are sensitive to clinically-relevant treatments and show clinical complications that are both relevant and ethically acceptable.

In summary, a relatively wide range of successful models for CIM have been developed using both tumor-bearing and healthy animals. The wide range of underlying diseases (cancers), patient phenotypes, chemotherapy regimens and supportive treatments used for tumor suppression in humans, makes it problematic to use only one animal model of CIM to advance both basic understanding and
clinical management of CIM. The GIT toxicity in CIM is highly dynamic, tissue- and species-specific. Nevertheless, animal models of CIM have lead to a vast amount of basic knowledge about CIM that help to understand which treatments have potential for human patients. Basic information about CIM obtained from animal models is an important foundation for the current understanding of CIM pathophysiology, prevention and treatment.
References


51


Whitford EJ, Cummins AG, Butler RN, Prisciandaro LD, Fauser JK, Yazbeck R, Lawrence A, Cheah KY, Wright TH, Lymn KA, and Howarth GS. Effects of Streptococcus


**Figure legends**

**Figure 1.** Translational research using animals is important as a "bridge" between basic biological understanding of chemotherapy-induced mucositis (CIM) and clinical treatment of CIM patients. Factors related to both basic and clinical research often challenge translation of animal model results to clinical treatments for patients. Regardless, crossing the bridge between science and clinics is important to facilitate new learning in both areas.

**Figure 2.** Limitations and potentials in basic versus clinical research related to CIM. Cell and rodent studies allow the best experimental control and mechanistic information while large animal models, and especially human studies, have the highest clinical relevance. Rodents have the best range of available laboratory analytical tools but large animal models better allow for detailed (e.g. GIT) sample collections and clinically-relevant interventions.

**Figure 3.** Characteristics of CIM studies in piglets (150, 167). Animals are kept individually in cages with full visual contact (A) and fitted with catheters for enteral and/or parenteral nutrition, chemotherapy, supportive drugs, antibiotics and repeated blood sampling (B). Protective clothing and careful disposal of the toxic chemotherapy-related waste material are required. After chemotherapy, macroscopic signs of CIM with varying intensity (blue arrow heads) can be observed in the oral cavity (C, D), colon (E), and small intestine (F-K). Severe lesions can be observed outside (F) or inside the intestine (G, H), while milder lesions are observed macroscopically as haemorrhagic spots along the mucosa (I, J), relative to the healthy intestinal mucosa (K).
Better understanding of CIM in both science and clinics

Answer scientific questions

Answer clinical questions

Crossing the bridge from animal models to human clinical CIM:

- Different gut anatomy, physiology, microbes, immunology
- Different age, geno-/phenotype, disease progression
- Differential biomarkers and tissue reactions
- Single- versus multiple-drug treatments
- Interactions with the background tumors
- Ethical, practical, economical limitations
**Research control & tools**
Mechanistic and hypothesis-generating

**Clinical relevancy**
Applied hypothesis-testing

**Ease of intervention and sample collection**

---

**Fig 2**

- **Basic research**
  - Cell models
  - Mice
  - Large rodents

- **Clinical research**
  - Larger animals
  - Human patients
Table 1. Overview of commonly used animal models for chemotherapy-induced mucositis (CIM)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cytotoxic regimens</th>
<th>Typical outcome measures</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats with or without cancer</td>
<td>MTX, 5-FU, Ara-C, cyclophosphamide, melphalan, doxorubicin, ironotecan, irradiation. Single-dose/repeated i.p., s.c., i.m or i.v.</td>
<td>Diarrhea, weight loss, intestinal digestion, absorption, bacterial translocation, histology, inflammation, apoptosis, proliferation, hematology.</td>
<td>(23, 27, 59, 67, 124)</td>
</tr>
<tr>
<td>Mice with or without cancer</td>
<td>MTX, 5-FU, AraC, melphalan, doxorubicin, vincristine, carboplatin, cyclophosphamide, irradiation. Single-dose/repeated i.p or i.m</td>
<td>Diarrhea, weight loss, intestinal digestion, absorption, bacterial translocation, histology, inflammation. Oral mucositis for irradiation models</td>
<td>(30, 35, 51, 124, 146, 212)</td>
</tr>
<tr>
<td>Hamsters without cancer</td>
<td>5-FU, irradiation. Single-dose/repeated i.p. or i.m</td>
<td>Oral mucosal ulceration, inflammation, growth factors</td>
<td>(26, 37, 117, 124, 174)</td>
</tr>
<tr>
<td>Pigs without cancer</td>
<td>5-FU, doxorubicin, cyclophosphamide, busulfan, irradiation. Single-dose/repeated, s.c. or i.v.</td>
<td>Clinical symptoms, vomiting, diarrhea, weight loss, digestion, absorption, intestinal histology, permeability, inflammation. Oral mucosal lesions.</td>
<td>(107, 128, 130, 150, 167, 201)</td>
</tr>
<tr>
<td>Dogs, cats, rabbits, with/without cancer</td>
<td>Cisplatin, irradiation, single-dose, repeated, cycles i.p., s.c., i.m, i.v.</td>
<td>Nausea, vomiting, gastric mucosal injury, oxidative stress, liver damage.</td>
<td>(13, 75, 129, 219)</td>
</tr>
</tbody>
</table>

Abbreviations: 5-fluorouracil (5-FU), cytosine arabinoside (Ara-C), methotrexate (MTX), intramuscular (i.m.), intraperitoneal (i.p.), intravenous (i.v.), subcutaneous (s.c.)
Table 2. Experimental GIT- and nutrition-related interventions in animal models of CIM

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Subjects</th>
<th>Findings</th>
<th>Conclusion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elemental nutrients</td>
<td>Rats, Cats</td>
<td>Diarrhea and vomiting↑, bacteria translocation↓, villus blunting↑, survival↓</td>
<td>Detrimental</td>
<td>(15, 129)</td>
</tr>
<tr>
<td>Whey extracts (+TGF-β, glutamine, casein)</td>
<td>Rats, hamsters</td>
<td>GIT growth↑, body weight↑, sucrase activity↑, GIT permeability↓, glutathione↑</td>
<td>Beneficial</td>
<td>(23, 37, 85, 195)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Rats, hamsters</td>
<td>Proliferation↓↑, intestinal surface↑, GIT permeability↓</td>
<td>Protective/detrimental</td>
<td>(38, 196)</td>
</tr>
<tr>
<td>Bovine colostrum</td>
<td>Pigs</td>
<td>Digestive function↑, GIT injury↓</td>
<td>Beneficial</td>
<td>(130, 148, 166)</td>
</tr>
<tr>
<td>PUFA (n-3)</td>
<td>Rats</td>
<td>Villus heights↑, proliferation↑, intestinal weight↑</td>
<td>Minimal benefits</td>
<td>(106, 193)</td>
</tr>
<tr>
<td>SCFA</td>
<td>Mice</td>
<td>Villus height↑, inflammation↓, necrosis↓</td>
<td>Reduction of mucositis</td>
<td>(152)</td>
</tr>
<tr>
<td>Emu oil PUFA</td>
<td>Rats</td>
<td>Villus height↑, inflammation↓</td>
<td>Beneficial</td>
<td>(106)</td>
</tr>
<tr>
<td>Supplementation</td>
<td>Organism</td>
<td>Effect on GI</td>
<td>Type of Effect</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>--------------</td>
<td>----------------</td>
<td>-------</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Rats, hamsters</td>
<td>Survival↑, tumor size↓, inflammation↓, ulceration↓, diarrhea↓</td>
<td>Beneficial</td>
<td>(104, 215)</td>
</tr>
<tr>
<td>Probiotics</td>
<td>Rats</td>
<td>Digestive function↑, permeability, apoptosis↓, weight loss↓</td>
<td>Prevent intestinal barrier disruption, moderate benefits</td>
<td>(175, 204, 210)</td>
</tr>
<tr>
<td>Prebiotics</td>
<td>Rats</td>
<td>GIT mass↑, villus and crypt↑</td>
<td>Beneficial</td>
<td>(216)</td>
</tr>
<tr>
<td>TGF-β, arginine, leptin</td>
<td>Rats, mice</td>
<td>Mucosal weight↑, GIT injury↓, villus and crypt↑, proliferation↓</td>
<td>Protective before/during chemotherapy</td>
<td>(17, 80, 118, 196)</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Rats</td>
<td>Proliferation↑, histological status↑</td>
<td>Improves healing</td>
<td>(186)</td>
</tr>
<tr>
<td>GLP-1 GLP-2</td>
<td>Rats, mice</td>
<td>Epithelial injury↓, intestinal repair↑, mucosal mass↑, sepsis↓</td>
<td>Protective against injury</td>
<td>(25, 102, 103, 189)</td>
</tr>
<tr>
<td>Multi-vitamins, riboflavin, folate</td>
<td>Rats</td>
<td>Oxidative stress↓, apoptosis↓</td>
<td>Attenuates GIT injury</td>
<td>(22)</td>
</tr>
<tr>
<td>Antioxidants (grape seeds)</td>
<td>Rats</td>
<td>Crypt depth↑, histological status↑, inflammation↓</td>
<td>Prevents GIT damage</td>
<td>(33)</td>
</tr>
</tbody>
</table>